

## FINAL REPORT

**TITLE:** GRAVITY AND SKELETAL GROWTH, NCC2-589

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This cooperative agreement was completed on December 31, 1998. It was first awarded on November 1, 1988. Though out the history of the agreement, yearly progress reports were submitted. The final year of the agreement was a no-cost extension to complete data aalysis and submit the mRNA data from the hindlimb unloading studies for peer-review. The paper was submitted to the Journal of Applied Physiology and has been accepted pending some minor revisions. A copy of the paper is attached.

**Invention Disclosure:** No inventions are anticipated from any of the research from this cooperative agreement.

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RESTORATION OF NORMAL WEIGHT BEARING FOLLOWING SPACEFLIGHT  
AND HINDLIMB UNWEIGHTING RESULTS IN TRANSIENT SITE-  
AND GENE-SPECIFIC CHANGES IN mRNA LEVELS  
IN LONG BONES OF GROWING MALE RATS

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**Key Words:** weightlessness, rat bone, TGF- $\beta$ , IGF-1, collagen and osteocalcin, bone formation

## ABSTRACT

Two simultaneous experiments were performed using 5-week-old male Sprague Dawley rats; in one study, the rats were flown in low earth orbit; in the other study, the hindlimbs of the growing rats were elevated to prevent weight bearing. Following 9 d of unloading, weight bearing was restored for 4, 28, and 76 hrs. Afterwards, additional hindlimb unloading experiments were performed to evaluate the skeletal response to 0, 2, 4, 6, 8, 10, 12, 16, and 24 hrs of restored weight bearing following 7 d of unloading. Cancellous and cortical bone histomorphometry were evaluated in the left tibia at the proximal metaphysis and in the left femur at mid-diaphysis, respectively. Steady-state mRNA levels for bone matrix proteins and skeletal signaling peptides were determined in total cellular RNA extracted from trabeculae from the right proximal tibial metaphysis and periosteum from the right femur. Spaceflight and hindlimb unloading each resulted in cancellous osteopenia, as well as a tendency towards decreased periosteal bone formation. Both models for skeletal unloading resulted in site specific reductions in mRNA levels for transforming growth factor- $\beta_1$  (TGF- $\beta$ ), osteocalcin (OC), and prepro- $\alpha$  (I) subunit of type 1 collagen (collagen) and little or no changes in mRNA levels for glyceraldehyde-3-phosphate dehydrogenase (GAP) and insulin-like growth factor I (IGF-I). Restoration of normal weight bearing resulted in transient increases in mRNA levels for the bone matrix proteins and TGF- $\beta$  in the proximal metaphysis and periosteum and no changes in either GAP or IGF-I mRNA levels. The timecourse for the response differed between the two skeletal compartments; the tibial metaphysis responded much more quickly to reloading. These results suggest that the skeletal adaptation to acute physiological changes in mechanical usage are mediated, in part, by changes in mRNA levels for bone matrix proteins and TGF- $\beta$ .

## INTRODUCTION

The overall effects of reduced mechanical usage on the human skeleton are characterized by a rapid reduction in mass and strength of weight bearing bones (15). Osteopenia has repeatedly been observed in laboratory animal models for disuse, including spaceflight (5,12,20,31,32), unilateral sciatic neurotomy (34), tendonotomy (26), hindlimb unloading (11), and immobilization by casting (18). The primary mechanism for the reduction in bone volume in humans and laboratory animals is a decrease in bone formation (1,20,28,38). Transient increases in bone resorption have been reported in some disuse models and may contribute to bone loss, especially in estrogen deficient female rats (5,26).

Understanding the signal transduction pathways that couples mechanical strain energy to bone cell number and activity might facilitate the development of novel approaches to prevent disuse-associated bone loss. Nevertheless, this pathway is poorly defined. Skeletal signaling peptides (growth factors and cytokines) including insulin-like growth factor I (IGF-I) and transforming growth factor- $\beta_1$  (TGF- $\beta$ ), have been implicated as intracellular signaling peptides in bone and other organs. These signaling peptides have been ascribed roles in mediating normal bone remodeling as well as skeletal adaptation to systemic and local factors (4,21).

Reductions in steady-state mRNA levels for TGF- $\beta$  were detected in extracted total cellular RNA from hindlimb periosteal following 8 and 11 days of spaceflight (35) as well as 14 days following unilateral sciatic neurotomy (Turner RT, unpublished results). Additionally, mechanical loading increases TGF- $\beta$  mRNA levels in bone cell culture (16). In contrast, the effects of loading on IGF-I expression may be complex. Steady-state mRNA levels for IGF-I were increased in the hind limb during unloading (3), whereas mechanical stimulation of tail vertebrae also resulted in an increase in IGF-I expression in vertebrae (17).

Bone is a major reservoir of TGF- $\beta$  and IGF-I; both growth factors are known to affect cell proliferation and differentiation in cell culture (4,21) and in skeletal tissues (14,19). It is, therefore, possible that these peptides may act as intermediates in the transduction pathway whereby bone cell metabolism is influenced by a mechanical stimulus. If this is the case, then one might expect that the expression of one or both of these signaling peptides would be altered in response to restoration of weight bearing to a previously unloaded limb.

The purpose of the present investigation was to perform time-course experiments to determine the progressive effects of renewed weight bearing on steady-state mRNA levels for signaling peptides and bone matrix proteins in long bones. Orbital spaceflight and hindlimb unloading were concomitantly evaluated to further validate the hindlimb unloading model for simulating the skeletal effects of spaceflight. Additionally, hindlimb unloading studies were performed to determine the early effects of restored weight bearing following skeletal unloading.

## Methods and Materials

### Animals

#### *Concurrent Spaceflight and Hindlimb Unloading Studies*

Seventy 5-week-old male Sprague Dawley rats were stratified by weight then randomly assigned to one of five groups: 1) Flight - 9 d in orbital spaceflight on the space shuttle *Discovery*, NASA mission STS-56. The flight rats were group housed (8 rats/group; n=16) in two animal enclosure models (AEMs); 2) Hind Limb unloaded - individually housed and hindlimb elevated for 9 d by the method of Wronski and Morey-Holton (N=16) (37), 3) Flight control - group housed in 2 AEMs (8 rats/group; n=16); 4) Vivarium control - housed in individual cages (N=16); and 5) Basal control - individually housed in vivarium cages until launch (N=6).

The rats were administered declomycin and calcein 7 and 1 d prior to launch, respectively. Tetracycline was given the day of landing to the rats that were sacrificed 1 and 3 d post landing.

The basal control group of rats was sacrificed immediately following launch to provide baseline measurements. Groups of animals (flight and hindlimb unloaded) were sacrificed by

anesthetization and exsanguination at 4, 28, and 76 hrs after restoration of normal weight bearing. Due to logistical constraints associated with moving animals from the orbiter to the laboratory, the time interval between the reloading of flight rats (reloading begins at reentry burn to bring the shuttle out of low earth orbit) and sacrifice was 4 hrs. Flight and vivarium control animals were euthanized at the same 3 timepoints. The periosteal cells were isolated from both excised femora of flight and flight control rats as previously described (35). Briefly, the bones were incubated for 2 hrs at 37°C with collagenase (2 mg.ml<sup>-1</sup>; Worthington Type II) in Hanks Balanced Salt Solution. Samples were centrifuged, washed twice in clean buffer to obtain a pellet of enriched periosteal cells, and frozen in liquid nitrogen. The collagenase-treated femora were rinsed with phosphate-buffered saline and fixed in 70% ethanol for cancellous bone histomorphometry. Trabeculae from the right proximal tibial metaphysis were excised as described, and frozen in liquid nitrogen (5). The left tibiae were fixed in 100% ethanol for cancellous bone histomorphometry while the left femora were fixed for cortical bone histomorphometry.

#### *Short-term Hindlimb Reloading Studies*

Additional hindlimb unloading experiments were performed in order to evaluate shorter intervals of reloading. These studies were identical to the first experiment except that the rats were hindlimb unloaded for 7 d and reloaded for 0, 2, 4, 6, 8, 10, 12, 16, and 24 hrs. The unloaded and reloaded rats were compared to time-matched weight-bearing controls to adjust for possible diurnal variations.

#### **Total RNA Isolation**

Total cellular RNA from the periosteum or trabeculae of bones pooled from 2-3 animals were isolated using a modified organic solvent method (7). Homogenization of the tissue was accomplished with a Tekmar TissueMizer (Cincinnati, OH) or Spex Freezer Mill (Edison, NJ) for periosteum and cancellous bone, respectively.

Total RNA yields from femora periosteum (average yield = 15-20 µg) and proximal tibia metaphysis (average yield = 60-80 µg) were determined spectrophotometrically at 260 nm. Ten µg

of each sample was denatured by incubation at 52°C in a solution of 1 M glyoxal, 50% dimethylsulfoxide in 0.1M NaH<sub>2</sub>PO<sub>4</sub> solution, then separated electrophoretically in a 1% agarose gel. The amounts of total RNA loaded and transferred were respectively assessed by ethidium bromide staining of the gels and hybridization of the filters with a <sup>32</sup>P labeled cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAP). GAP was unchanged with either spaceflight or hindlimb unloading.

### **Northern Blot Analysis**

The RNA was transferred overnight via capillary action in 20X SSC buffer to an Amersham Hybond nylon membrane and cross-linked with a Stratagene UV Stratalinker 1800 before hybridization. The filters were prehybridized for 6 hrs at 45°C in a buffer containing 50% deionized formamide, 10% dextran sulphate, 5X SSC (1X SSC = 0.15 M NaCl, and 0.015 M Na Citrate, pH 7.0), 600 µg/ml heat denatured, single-stranded salmon sperm DNA, and 2X Denhardt's solution. Hybridization was carried out for 18 hrs in a buffer containing the above ingredients in addition to 10<sup>6</sup> cpm ml<sup>-1</sup> <sup>32</sup>P labeled cDNA for the various probes. cDNA probes were labeled by random sequence hexanucleotide primer extension using the Amersham Megaprime DNA labeling kit (Arlington Heights, IL). The filters were washed for 30 min at 45°C in 2X SSC and between 15-60 min at 0.1X SSC at 45°C. The mRNA bands were quantitated by densitometric scanning using a Molecular Dynamics Phosphor Imager (Sunnyvale, CA).

The cDNA probes used were 1) rat TGF-β cloned in pBluescript IIKS+ vector. This fragment is excisable with Hind III and XbaI and was provided by Dr. MJ Sporn at the National Cancer Institute, National Institutes of Health; 2) human IGF-I, a gift from Dr. G. Bell, Howard Hughes Medical Institute Research Laboratories, University of Chicago (Chicago, IL); this plasmid p<sub>h</sub><sub>13</sub>F1 containing an insert of about 660 bp which extends from the second nucleotide of the codon for amino acid-15 of the single peptide of the IGF-I precursor to the poly(A) tract (2); 3) Rat GAP was a gift from Dr. P Fort, Laboratoire de Biologie Moléculaire, Montpellier, France. This pUC18 plasmid DNA contains the full length rat GAP cDNA inserted at the Pst I site (9); 4)

rat OC was a gift from Dr. S Rossi-Langen, Genetics Institute, Cambridge, MA (6); 5) rat collagen, a gift from Dr. C Genovese, University of Connecticut, Farmington, CT (10).

### **RNase Protection Assay for IGF-1 mRNA**

The RNase protection procedure was performed on RNA from proximal tibial metaphyses using the RPA II kit (Ambion, Austin, TX) as recommended by the manufacturer. Briefly, a 226 bp fragment of rat IGF-I cDNA was synthesized by reverse transcriptase polymerase chain reaction from rat liver RNA using oligonucleotide primers with the following sequences: CAGAATTCGCCGGACCAGAGACCCTTTG and CAGGATCCCCCGGATGGAACGAGCTGAC. These primers contain EcoRI and BamHI sites and 20 bp sequences from exons 3 and 4 of the rat IGF-1 gene, respectively. The resultant cDNA fragment was cloned into the EcoRI and Bam HI sites of the SK Bluescript vector (Stratagene). A 293 nucleotide antisense RNA probe was synthesized and labeled with  $^{32}\text{P}$ -UTP by in vitro transcription using 100 ng of IGF-SK vector (linearized with EcoRI) and T3 polymerase by the method of Kreig, 1990. Similarly, a GAP antisense probe was synthesized using the provided template (Ambion). The antisense RNA probes were purified by DNase digestion of the template followed by G-50 gel filtration. The purified antisense RNA probes ( $6 \times 10^4$  cpm) were hybridized for 18 hr at 43°C in 20  $\mu\text{l}$  of hybridization buffer (80% deionized formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4, 1 mM EDTA) containing bone RNA (10  $\mu\text{g}$  for IGF-I and 5  $\mu\text{g}$  for GAP). The hybridized RNA was digested with 0.5 units of RNase A and 20 units of RNase T1 for 30 min at 37°C, then ethanol precipitated and resuspended in 8  $\mu\text{l}$  of gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 mM EDTA, 0.025% SDS). The RNase digested samples were then fractionated by urea-polyacrylamide (50% urea w/v, 7% acrylamide w/v, 1x TBE) gel electrophoresis. The polyacrylamide gel was vacuum dried and placed on a phosphorimager screen for 18 hrs. Quantitation of protected IGF-I RNA fragments was performed by phosphorimager analyses and normalized to GAP RNA levels.

### **Cancellous Bone Histomorphometry**

The proximal metaphysis from the left tibia was dehydrated in ascending grades of ethanol. The undecalcified bone was then infiltrated and embedded in a methylmethacrylate plastic mixture as described (36). The resulting blocks were sectioned at an indicated thickness of 5  $\mu\text{m}$  on a supercut 2050 microtome (Richert-Jung) to obtain midcoronal sections. A set of unstained sections were mounted with Eukitt.

Cancellous measurements were performed with semiautomatic image analysis systems under UV or visible light, using Osteomeasure software (Osteometrics; Atlanta, GA). Cancellous static and dynamic measurements were performed at optical magnifications of 9.8X and 18.75X, respectively. These measurements were taken from a standardized, growth-adjusted site within the metaphysis. This site was located 1 mm distal to the growth plate and excluded trabecular profiles connected to the endocortical surface.

The following measurements were obtained.

Cancellous Bone Area (B.Ar.): The area that consists of cancellous bone, expressed as % of metaphyseal tissue area.

Cancellous Bone Perimeter (B.Pm): The perimeter delineated by cancellous bone, expressed as  $\text{mm}/\text{mm}^2$  metaphyseal area.

Trabecular Thickness (Tb.Th):  $\text{Tb.Th} = 2/\text{B.Pm}/\text{B.Ar}$  (22)

Trabecular Separation (Tb.Sp):  $\text{Tb.Sp} = \text{Tb.Th}/(\text{B.Ar}/\text{T.Ar}) - \text{Tb.Th}$  where T.Ar is tissue area (22).

Trabecular Number (Tb.N):  $\text{Tb.N} = (\text{B.Ar}/\text{T.Ar})/\text{Tb.Th}$  (22)

### **Cortical Bone Measurements:**

Histomorphometric measurements were performed with the SMI-Microcomp-P.M. semiautomatic image-analysis system (Southern Micro Instruments, Inc., Atlanta, GA) as described (5). Ground transverse sections from the midshaft were prepared for histomorphometric analysis of

cortical bone as described (35). The following measurements were obtained; abbreviations conform to standardized nomenclature (23).

**Cross Sectional Area (Cs.Ar):** The total area (bone and marrow) within the periosteal surface (Ps.Pm) of the specimen, measured in  $\text{mm}^2$ .

**Medullary Area (Me.Ar):** The medullary cavity, delineated by the endocortical perimeter (Dp.Ec.Pm) of the specimen, measured in  $\text{mm}^2$ .

**Diaphyseal Bone Area (Dp.Ar):** The bone area at the diaphysis is a value calculated from the cross sectional area minus the medullary area. It represents the mineralized bone area in the cross section and is expressed as  $\text{mm}^2$ .

**Bone Formation Rate (BFR):** The area between the concentric fluorescent labels (or innermost fluorescent label and perimeter) on the periosteal margin of the cross sections divided by the number of days elapsed between administration of two fluorochrome labels (or final label and necropsy). Expressed as  $\text{mm}^2/\text{d} \times 10^{-3}$ .

**Mineral Apposition Rate (MAR):** The mean thickness (measured within the periosteal envelope) of newly formed mineralized bone expressed as a daily average, during the time period between fluorochrome label (or final label and necropsy) administrations. This value is calculated as MAR divided by the mean length (perimeter) of the two consecutive fluorochrome markers (or final fluorochrome label and periosteal perimeter) and is expressed as  $\text{mm}^2/\text{d} \times 10^{-3}$ .

### **Statistical Analysis:**

One-way ANOVAs were performed with Fisher protected least significant difference post-hoc multiple comparison tests to establish significance. The unloaded and reloaded groups were compared to the time- and cage-matched weight bearing controls.

## **RESULTS**

The rats tolerated the 9 day spaceflight well; they appeared healthy and there were no differences in the rate of weight gain relative to either ground control group.

The effects of 9 days of spaceflight and hindlimb unloading on cancellous bone architecture are shown in Table 1. Spaceflight and hindlimb unloading each resulted in cancellous osteopenia at the proximal tibia metaphysis. Cancellous bone area and trabecular number were decreased and trabecular separation was increased compared to the weight bearing baseline controls, time-matched flight controls and/or time matched vivarium controls, whereas there were no changes in trabecular thickness.

When compared to the weight bearing flight control and vivarium groups, 9 days of spaceflight and hindlimb elevation each resulted in a nonsignificant tendency toward decreases in the mean periosteal bone formation and mineral apposition rates, and increases in the endocortical bone formation and mineral apposition rates (Table 2). As expected in short-term studies, neither spaceflight nor hindlimb unloading influenced the cross sectional, medullary and cortical bone areas.

Representative phosphorimages of Northern hybridizations for collagen, OC, TGF- $\beta$ , and GAP are published (1,35). Each of the mRNAs were detected by Northern analysis in long bone periosteum and proximal tibial metaphysis. IGF-I mRNA was detected by Northern analyses in total cellular RNA isolated from periosteum but was not detected using this method in the RNA isolated from the metaphysis. However, IGF-I was easily detected in distal femur metaphysis by solution hybridization using an RNA probe (5).

Neither spaceflight nor hindlimb unloading had effects on mRNA levels for either GAP or IGF-I at either skeletal site (data not shown).

The effects of unloading and time course effects of reloading on steady-state mRNA levels for bone matrix proteins and TGF- $\beta$  are shown in Figure 3.

Differences in mRNA levels for TGF- $\beta$  and OC were not detected in the proximal tibial metaphysis following restoration of normal weight bearing for 4, 28, and 76 hrs after either spaceflight or hindlimb unloading (Figure 3a). No consistent response was observed at that site for collagen; reloading following spaceflight resulted in a tendency toward a modest decrease in message levels whereas reloading following hindlimb unloading resulted in an increase.

In femoral periosteum, hindlimb unloading resulted in decreases in the steady-state mRNA levels for TGF- $\beta$  (-29%), OC (-64%) and collagen (-63%) (Figure 3b). Reloading following spaceflight resulted in large increases in message levels for all 3 genes at 28 hr (TGF- $\beta$ , 435%; OC, 808%; collagen, 992%) followed by a return to reduced (collagen and OC) or normal (TGF- $\beta$ ) message levels at 76 hr. Reloading following hindlimb unloading resulted in a qualitatively similar, but attenuated, (TGF- $\beta$ , 345%; OC, 486%; collagen, 686%) response at 28 hrs.

#### *Short-term Hindlimb Reloading Studies*

The subsequent hindlimb elevation studies demonstrated that reloading results in rapid (4-12 hrs), transient increases (175-300% weight bearing control) in mRNA levels for bone matrix proteins and TGF- $\beta$  followed by a return to normal values. Compared to the tibial metaphysis, the changes in mRNA levels at the periosteum occurred later. Additionally, there appeared to be gene specific differences in the time course response to reloading. The steady-state mRNA levels for the matrix proteins increased gradually with time, whereas TGF- $\beta$  showed an abrupt increase after 16 hr of reloading. Collagen mRNA levels increased progressively during the first 24 hrs of reloading. In contrast, OC expression demonstrated a more complex response.

### DISCUSSION

Spaceflight and hindlimb unloading resulted in changes at the skeletal sites where RNA was isolated for analysis of gene expression. Most notably, periosteal bone formation appeared to be slightly depressed at the femur mid-diaphysis and there was a net reduction in cancellous bone volume at the distal metaphysis. mRNA levels for bone matrix proteins were reduced by 25-70% compared to the weight bearing flight controls. Restoration of weight bearing following spaceflight and hindlimb elevation each resulted in transient elevations in mRNA levels for selected bone matrix proteins and TGF- $\beta$ . The gene changes were site- and gene-specific; there were no changes in mRNA levels for either GAP or IGF-I, and the time course for the changes in mRNA levels for bone matrix proteins and TGF- $\beta$  differed between periosteum and cancellous bone.

The histomorphometric and gene changes following unloading are in general agreement with previous studies (1,35). However, it is difficult to make direct comparisons because of differences in age, strain and duration of flight among experiments. The decreases in periosteal bone formation in the 9 d spaceflight study failed to reach statistical significance. Previous short-term studies suggest that the inhibition of bone formation associated with spaceflight is not immediate (1,39). Since the fluorochrome label used to calculate bone formation was administered prior to launch, the calculated bone formation rate is an average over the 10 d interval, which includes 1 d of normal weight bearing. Thus, it is likely that the calculated bone formation rate overestimated the bone formation rate at the end of the flight period. The notable reduction in collagen and osteocalcin mRNA levels following spaceflight supports the conclusion that periosteal bone formation was reduced at the end of the spaceflight study.

A recent study suggests that group-housed young rats are less responsive to the inhibitory effects of weightlessness on periosteal bone formation than either older or individually housed rats (39). The animals used in this study were the same as in the present study. The time-course effects of hindlimb unloading and spaceflight may differ because of these factors and because of the temporary effects of the acceleration at launch and re-entry experienced by the flight rats. However, in the present studies similar results were obtained for all femora measurements regardless of the method used to unweight the bone. We did detect an inhibition of periosteal bone formation at the tibia-fibula synostosis in hindlimb elevated rats (data not shown) but not in spaceflight. This finding suggests that there may be site-specific differences.

Several studies have investigated the effects of spaceflight on cancellous bone histomorphometry in male rats (12,28,31,33,35). These studies, depending upon the duration of the flight and the bone studied, detected either no change in cancellous bone volume or osteopenia. The few studies in which dynamic measurements were performed suggest that spaceflight results in depressed bone formation with little or no accompanying change in bone resorption (28,33).

Whatever the cellular mechanism, the cancellous osteopenia was due to a decrease in trabecular number; there was no change in trabecular thickness.

The paucity of dynamic cancellous bone data is the direct result of limitations imposed by the AEM housing. There is no provision for administering fluorochrome labels during spaceflight. Additionally, weight limitations necessitate using young, rapidly growing rats in order to insure a sufficient sample size. As a consequence of rapid longitudinal bone growth and destructive bone modeling in young rats, fluorochrome labels given preflight often can no longer be detected following the flight (28,35,39). This was the case for the present study. This limitation is not a factor for cortical bone measurements because bones increase in cross sectional area by secondary intramembranous ossification, the rate of cortical bone remodeling is very low and periosteal resorption is relegated to developing vascular spaces.

Investigators have partially circumvented the limitations of performing spaceflight experiments in young rats. This has been accomplished by evaluating bones, such as the humerus (28), which undergo epiphyseal closure at an early age (13,28), by studying smaller more slowly growing strains of rats such as Fisher 344 (35), by using female rats which are smaller and grow more slowly than males of the same strain (5), and by using alternative indices for bone formation such as mRNA levels for type 1 collagen.

We have used steady-state mRNA levels for collagen as an index of the rate of bone formation immediately following spaceflight. As mentioned, mRNA levels for type 1 collagen were decreased following unloading. The rationale for this approach is based upon the knowledge that collagen is quantitatively the most important component of the organic matrix of bone (25), that there is a high correlation between the rate of bone formation measured by dynamic bone histomorphometry and collagen mRNA levels in rat bone (27,30), and that mRNA levels for collagen and histological indices of bone formation increase and decrease following treatment of rats with intermittent PTH (8) and estrogen (29), respectively. Additionally, <sup>3</sup>H-proline radioautography studies have revealed that most

of the collagen synthesized in rat long bones is produced by osteoblasts and is deposited into bone matrix (36).

Restoration of normal weight bearing following spaceflight resulted in a transient increase in mRNA levels for type 1 collagen. Similar transient increases in mRNA levels for OC and TGF- $\beta$  were observed. However, there were no changes in mRNA levels for IGF-I and GAP indicating that the changes are gene specific. Similar changes occurred in rats following hindlimb elevation or renewed weight bearing (24), suggesting that this response is due to decreased weight bearing rather than the method used to unweight the skeleton. This observation is important because hindlimb unloading is the more practical and convenient model to investigate the cellular and molecular response to reloading.

There is no consensus regarding the effects of mechanical usage on expression of IGF-I. External loading has been shown to result in rapid increases in mRNA levels for IGF-I in osteocytes of tail vertebrae (17). The rapid stimulatory effect of loading on IGF-I mRNA levels reported in the osteocyte contrasts with a study showing that bones from unweighted hindlimbs expressed higher message levels for IGF-I (3). The negative results obtained in the present study suggest that re-establishment of normal weight bearing to an unloaded limb has no acute tissue level effects on IGF-I expression in either periosteum or cancellous bone.

The apparent lack of response of the proximal tibial metaphysis to reweighting following spaceflight and hindlimb elevation was a major surprise because histomorphometric changes were observed at this site in a variety of unloading models (11,26,34,35). However, the subsequent short-term reloading studies have revealed that mRNA levels for bone matrix proteins and TGF- $\beta$  are significantly reduced in cancellous bone of an unloaded limb but that these message levels increase more rapidly following reloading than in periosteum. Thus, our failure to detect changes in message levels in cancellous bone following spaceflight was probably due to the timing of the harvesting the tissues.

In summary, the present study demonstrates that there are important compartment-specific differences in the timing of the skeletal response to a mechanical loading. The mechanism for this

difference is unknown. Our results suggest physiological loading results in rapid, transient site- and gene-specific increases in expression of specific bone proteins. These results further suggest alterations in expression of genes for matrix proteins and skeletal signaling peptides are important intermediates in the signaling pathways by which mechanical usage influences bone volume.

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Table 1. The effects of spaceflight and hindlimb unloading on cancellous bone architecture in the proximal tibial metaphysis					
Measurement	Baseline	Flight Control	Flight	Hindlimb Unloaded	Vivarium
BV/TV (%)	11.0±1.5	9.9±.9	6.7±.6*	7.0±.3*	11.0±.9
Tb.Th (µm)	40.0±2.1	40.0±1.6	35.0±1.7	36.7±1.6	39.2±1.5
Tb.N (mm <sup>-1</sup> )	2.7±.3	2.5±.2	1.9±.2*	2.0±.1*	2.8±.2
Tb.Sp (µm)	39.5±52	434±35	613±58*	535±33*	378±24
Values are mean ± SE; (N=6 for baseline and 16 for all other groups). Bone volume (BV); tissue volume (TV); trabecular thickness (Tb.Th); trabecular number (Tb.N); Trabecular separation (Tb.Sp). * p < .05 compared to baseline values.					

Table 2. The effects of spaceflight and hindlimb unloading on cortical bone histomorphometry in the distal femur				
Measurement	Flight Control	Flight	Hindlimb Unloaded	Vivarium
Cross sectional area (mm <sup>2</sup> )	6.81±.19	6.95±.15	6.59±.25	6.88±.20
Medullary Area (mm <sup>2</sup> )	3.02±.14	3.23±.09	2.95±.26	2.98±.16
Cortical Area (mm <sup>2</sup> )	3.79±.11	3.72±.06	3.64±.15	3.89±.08
Ps.BFR (mm <sup>2</sup> ×10 <sup>-3</sup> /d)	104±9	89±3	87±11	97±7
Ps.MAR	13.0±1.3	11.6±.9	12.5±1.0	10.9±.7
Ec.BFR	19.7±2.0	24.6±.3	23.0±3.3	16.8±1.6
Ec.MAR	3.9±.3	4.5±.6	3.7±.3	2.9±.2
Values are mean ± SE (N=5-6). * P < .05 compared to flight control. Periosteal (Ps); endocortical (Ec); bone formation rate (BFR); mineral apposition rate (MAR)				

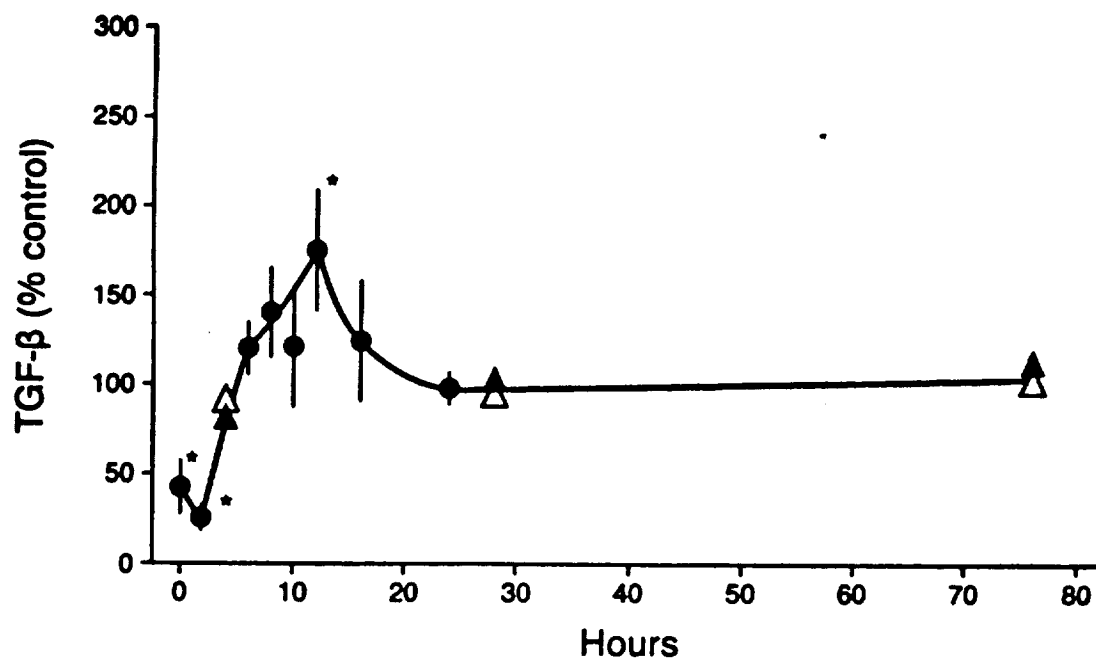
## FIGURE LEGENDS

Figure 1.--The effects of unloading and reloading on steady-state mRNA levels for collagen, (A. proximal tibial metaphysis; B. femur periosteum), OC (C. proximal tibial metaphysis; D. femur periosteum) and TGF- $\beta$  (E. proximal tibia metaphysis; F. femur periosteum).

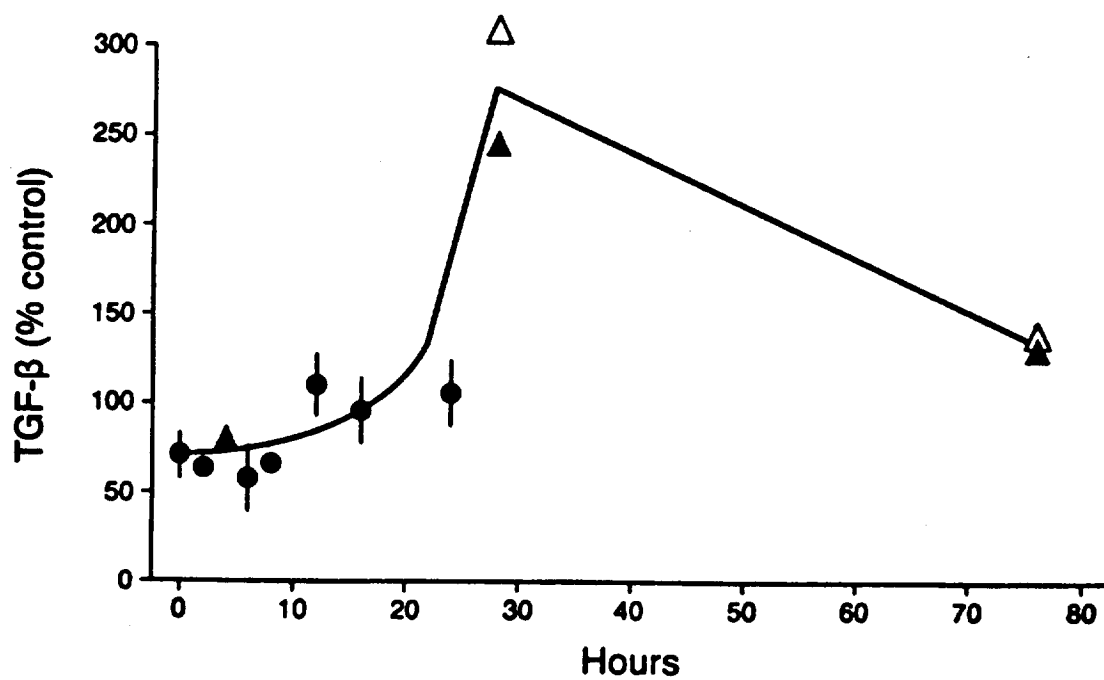
▲ and Δ indicate the mean values for rats flown in space or subjected to the concurrent hindlimb unloading study, respectively. These values usually represent the mean of single measurements from two separate pools (N=2). Each pool consisted of RNA isolated from femora from 2-3 rats.

● indicates the mean values for the subsequent short-term hindlimb unloading studies. The error bars represent  $\pm$  SE; n=3 (periosteum) or 6 (proximal metaphysis). RNA was isolated from individual bones (proximal femur metaphysis) or bones pooled from 2 animals (proximal femur/metaphysis). \* is  $p < .05$  compared to weight bearing controls.

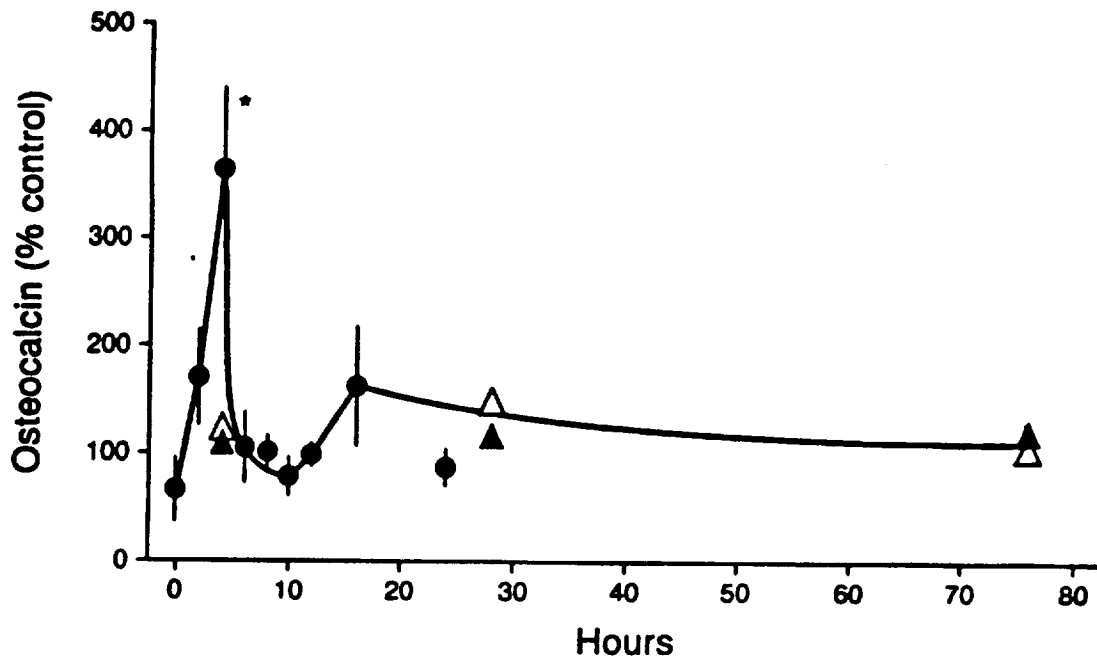
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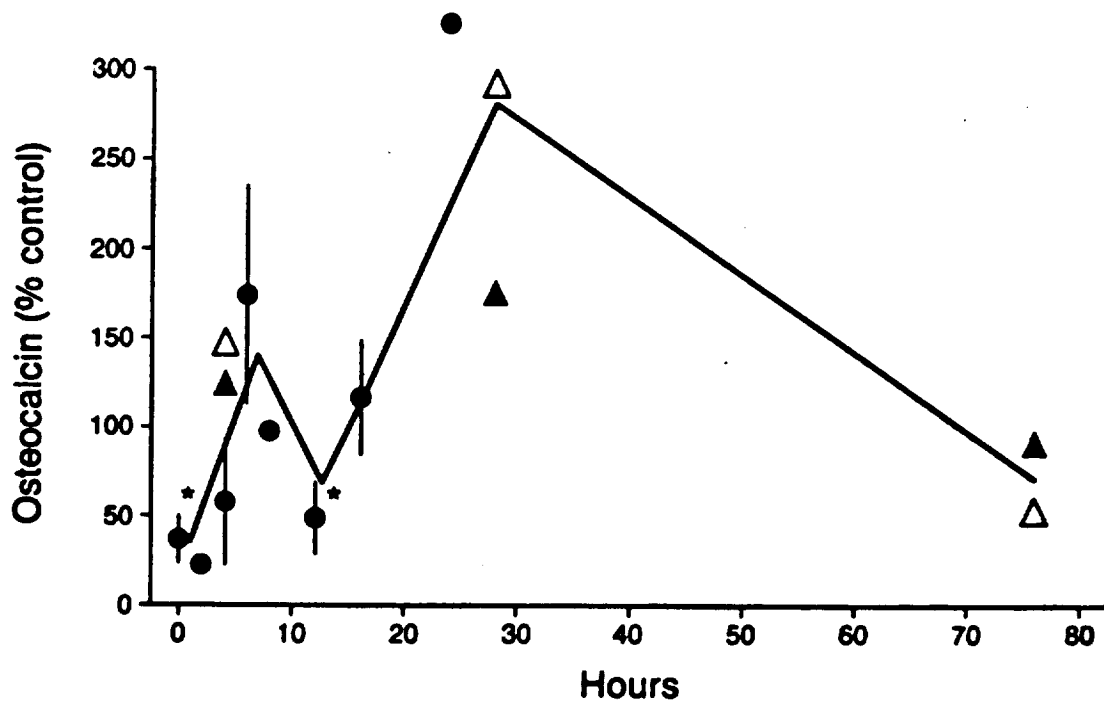
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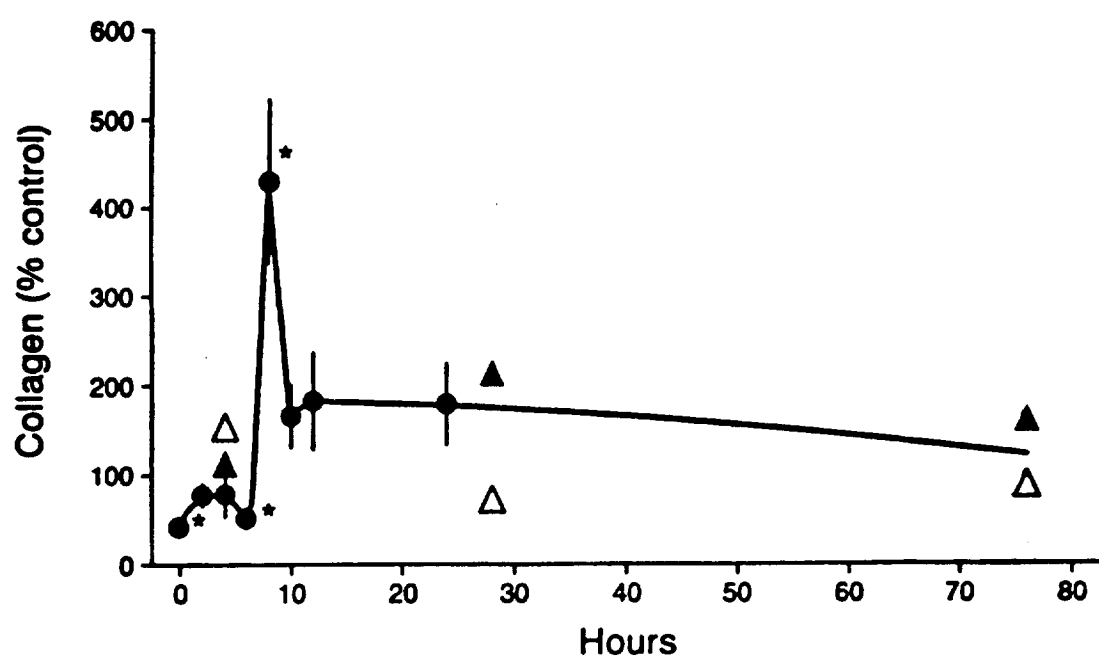
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D



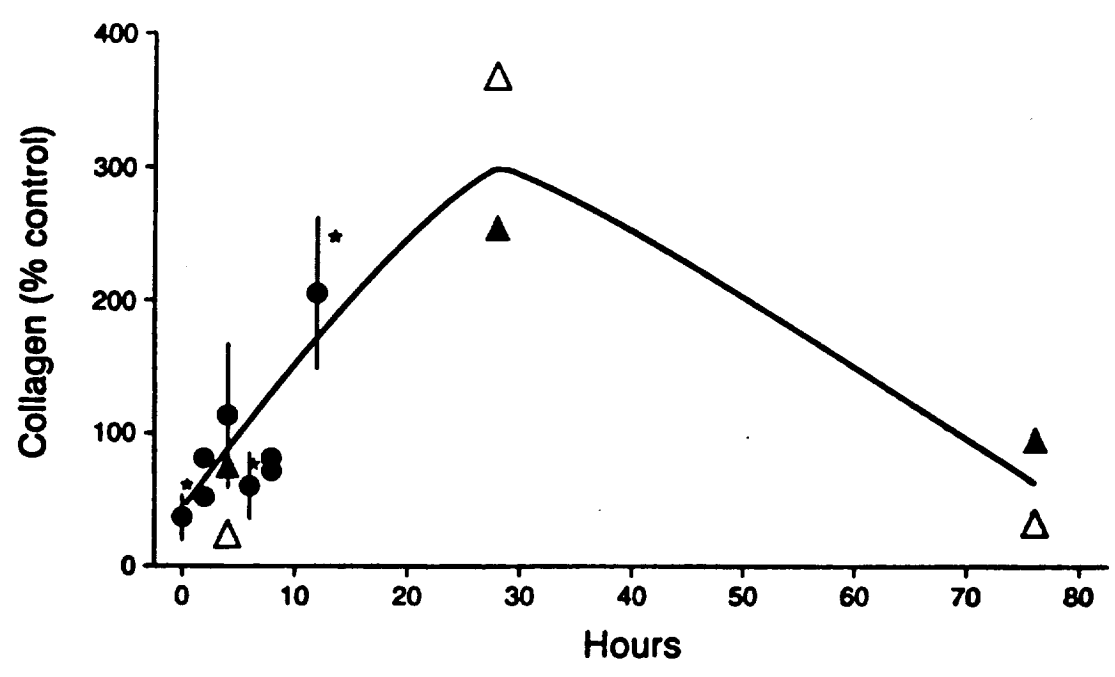
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III



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IV



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